The development of antimicrobial biomaterial surfaces

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Positively charged biomaterials exert antimicrobial effects on Gram-negative bacilli in rats

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Biomaterial-centered infection is a much-dreaded complication associated with the use of biomedical implants. Although positively charged biomaterial surfaces stimulate bacterial adhesion, it has been suggested that surface growth of adhering Gram-negative bacilli is inhibited on positively charged surfaces. In the present paper, we determined the infection rate of differently charged poly(methacrylates) in rats. To this end, 2 x 10^6 cm^{-2} *Escherichia coli* O2K2 or 2 x 10^4 cm^{-2} *Pseudomonas aeruginosa* AK1 were seeded on glass discs coated with three differently charged poly(methacrylates) coatings in a parallel plate flow chamber. Three rats received six subcutaneous discs (two discs of each charge variant) seeded with *E. coli*, while three other rats received discs seeded with *P. aeruginosa.* The numbers of viable bacteria on the surfaces were determined 48 h after implantation. On 50% of all positively charged discs viable *E. coli* were absent, while the negatively charged discs were all colonized by *E. coli*. *P. aeruginosa*, however, were isolated from both positively and negatively charged discs. Probably, *P. aeruginosa* can circumvent the antimicrobial effect of the positive charge through the formation of extracellular polysacharides.

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Introduction

Infection is the most common cause of biomaterial implant failure in modern medicine [1,2]. Adhesion and subsequent surface growth of bacteria on biomedical implants and devices causes the formation of a biofilm, in which the so-called "glycocalix" embeds the infecting bacteria, and offers it protection against the host immune system and antibiotic treatment [3]. Recently, we found that adhering *Escherichia coli* O2K2 and *Pseudomonas aeruginosa* AK1 were unable to grow *in vitro* on poly(methacrylates) containing positively charged quaternary ammonium groups [4,5]. Although bacterial adhesion was initially lower on negatively charged poly(methacrylates), this effect was rapidly counterbalanced by surface-growth of the adhering bacteria. It was concluded, that whether biomaterial-centered infections occur under clinical conditions, depends on a critical balance between the effects of biomaterial surface properties on bacterial adhesion and their inverse effects on growth.

The aim of this study was to determine, within a homologous series of three methacrylate polymers and copolymers with varying surface charge, whether positively charged surfaces have a lower risk of becoming infected under *in vivo* conditions. To this end, *E. coli* O2K2 or *P. aeruginosa* AK1 were allowed to adhere on poly(methacrylates) with different surface charges, after which the materials were implanted subcutaneously in rats, and evaluated after 48 h for numbers of viable bacteria.

Materials and methods

Animals

Six male, 14 week old, specific-pathogen free Albino Oxford rats weighing 300 g were used. The animals were maintained under clean conventional conditions and fed standard rat chow and water *ad libitum*. The animals were allowed to acclimatize to our laboratory conditions for 2 weeks before experiments. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" (NIH Publication No.85-23, revised 1985) and the Dutch Law on Experimental Animal Care.

Bacteria

E. coli O2K2 was cultured in brain heart infusion in phosphate buffered saline (PBS) and *P. aeruginosa* AK1 in nutrient broth in PBS. First, a strain was streaked and grown overnight at 37°C from a frozen stock on a blood agar plate. A colony was used to inoculate 5 ml of growth medium, which was incubated at 37°C in ambient air for 24 h and used to inoculate a second culture (100 ml) that was grown for 18 h. The bacteria from the second culture were harvested by centrifugation and washed twice with sterile Millipore-Q water. Subsequently, the bacteria were resuspended in PBS. The concentration of bacteria was determined using a counting chamber and adjusted to 1 x 10^9 cells ml^{-1} in PBS.

Polymer synthesis

Polymers and polymer films were prepared as described previously [6]. Briefly, homopolymers of methyl methacrylate (PMMA) and copolymers of MMA with either 15 mol% methacrylic acid (PMMA/MAA 85/15) or 15 mol% trimethylaminoethyl methacrylate chloride (PMMA/TMAEMA-Cl 85/15) were synthesized by radical polymerization of the monomers using 2,2'-azobis(methyl isobutyrate) as an initiator. Glass discs (diameter 8 mm, 1 mm thick) were cleaned by immersion in a mixture of hydrochloric acid (37%) and nitric acid (65%), ratio 3:1 (v/v) for 20 h. After extensive rinsing with double de-ionized water and ethanol, the discs were dried *in vacuo* at 60 °C for 3 h. The discs were silanized with n-propyltrimethoxysilane in case of PMMA and with γ-aminopropyltriethoxysilane in case of PMMA/MAA. The polymer films were prepared on both sides of the discs by spin coating [6]. Finally, the polymer films were dried *in vacuo* at 60 °C for 18 h in sterile petridishes to remove any solvent remnants. The zeta potentials, indicative of the surface charge, of the polymer films in PBS were determined as described previously [7], and were –18 mV for PMMA/MAA, -12 mV for PMMA and +12 mV for PMMA/TMAEMA-Cl.

Seeding of bacteria and implantation.

The bacteria were seeded on the coated glass discs in a parallel plate flow chamber, according to a procedure that has previously been described in detail [8]. A bacterial suspension of 1 x 10^9 cells ml^{-1} in PBS was passed through the chamber at a flow rate of 0.025 ml s^{-1} for 1 h. Then, flow was switched to PBS without bacteria to remove unbound organisms from the tubes and the flow chamber under the same flow rate for 30 min. Phase-contrast microscope

images were taken of each polymer coating, and the total numbers of adhering bacteria were counted using image analysis software. Finally, the tubes were blocked and the chambers were opened. The discs were carefully removed and either put in 5 ml reduced transport fluid (RTF) for CFU determination or subcutaneous implantation in rats.

Before implantation, the backs of the rats were shaved and disinfected with chlorhexidine in 70% ethanol after induction of inhalation anesthesia with N_2O/O_2 (3/2) and halothane. Six 1 cm incisions were made, three on either side of the middle line, at least 2 cm apart. Subcutaneous pockets of at least 2 cm deep were created. At either side of the middle line three differently charged discs were inserted and the incisions were closed with 2 stitches. Three rats received discs seeded with *E. coli* and three others received discs seeded with *P. aeruginosa.* After 48 h the rats were terminated with CO_2. After skin disinfection and opening, the discs were carefully removed and added to 5 ml RTF for enumeration.

For enumeration, discs in RTF were first treated in a sonicating water bath for 10 min to remove adhering bacteria. Removal of the bacteria was verified with phase contrast microscopy. Then, suspensions were diluted and streaked on blood agar to determine the number of colony forming units (CFU). The lowest number that could be detected was 50 bacteria per disc. The infection incidence was defined as the percentage of explanted discs per charge variant from which bacteria could be harvested.

Table 1. Infection incidence (number of infected discs over the total number of discs involved) for a homologous series of subcutaneously implanted differently charged poly(methacrylates) and CFU ± standard errors (in 10^4 cm^{-2}) before and after 48 h of subcutaneous implantation in rats (two discs of each charge variant were implanted in three rats). Note that the CFU presented are averages, only pertinent to the infected discs

Bacterial strain	Parameter	PMMA/MAA (-- charge)	PMMA (- charge)	PMMA/TMAEMA-Cl (+ charge)
E. coli O2K2	Incidence	6/6	6/6	3/6
	CFU before	520 ± 280	960 ± 280	1440 ± 560
	CFU after	1.1 ± 0.3	70 ± 60	4.8 ± 1.9
P. aeruginosa AK1	Incidence	5/6	6/6	6/6
	CFU before	5.2 ± 1.2	9.2 ± 0.8	7.6 ± 1.2
	CFU after	5.2 ± 4.1	2.5 ± 1.1	36 ± 26

Results and discussion

Table 1 shows the infection incidence and the mean numbers of CFU on the infected materials before and after 48 h of implantation. The infection incidence of *E. coli* O2K2 was twice as low on the positively charged PMMA/TMAEMA-Cl than on the negatively charged surfaces as bacteria were recovered from only three discs. Moreover, the number of viable *E. coli* decreased significantly (Student t-test, $p<0.05$) during implantation, especially on PMMA/MAA and PMMA/TMAEMA-Cl. On the negatively charged PMMA/MAA and PMMA, the numbers of CFU for *P. aeruginosa* AK1 were similar before and after implantation. Unexpectedly, the number of CFU on positively charged PMMA/TMAEMA-Cl increased during 48 h of implantation, although this increase was not statistically significant (Student t-test, $p>0.05$). Note that no viable *P. aeruginosa* were isolated from one PMMA/MAA disc. Also the CFU on the discs before implantation were not significantly different between the three differently charged discs, although the total number of adhering bacteria (viable and non-viable) as determined by phase contrast microscopy was significantly higher on the positively charged discs (compared to PMMA 4 times for *E. coli* and 2 times for *P. aeruginosa*).

From these results, it can be concluded that positively charged poly(methacrylate) surfaces have a lower risk of becoming infected by *E. coli* O2K2 under *in vivo* conditions than negatively charged surfaces. As before implantation the numbers of viable *E. coli* were similar on all charged surfaces, this is probably due to physical inhibition of bacterial surface growth, as also observed *in vitro* [5]. Although *in vitro* inhibition of surface growth was also seen for *P. aeruginosa* AK1 on positively charged surfaces [4], results for this organism evidently do not hold *in vivo.* Possibly, *P. aeruginosa* AK1 produces considerably larger amounts of extracellular polysacharides than *E. coli* O2K2, creating a barrier between its cell surface and the antimicrobial positively charged surface. Extracellular polysacharides are known virulence factors in bacterial infection, protecting the organisms against the hosts immune system [9], which explains why *P. aeruginosa* AK1 can grow on the implanted discs, whereas *E. coli* were readily eradicated by the immune system of the rats, especially on the positively charged discs.

In conclusion, positively charged biomaterials with reduced surface growth of adhering bacteria have a lower risk of infection *in vivo* than negatively charged surfaces. The development of such antimicrobial surfaces may have several applications, for instance on

medical devices used in the urogenital tract, where catheter-associated infections are rampant [10,11] and *E. coli* constitute the most important causative organisms for infection [12]. Especially now that many antibiotics have become ineffective due to bacterial resistance [13,14], the application of positively charged surfaces may provide a clinical solution to several types of biomaterial-centered infections.

References

1. **Gristina, A.G. (1987).** Biomaterial-centered infection: microbial adhesion versus tissue integration. *Science* **237,** 1588-95.
2. **Verkerke, G.J., Schraffordt-Koops, H., Veth, R.P., Van Horn, J.R., Postma, L. and Grootenboer, H.J. (1997).** First clinical experience with a noninvasively extendable endoprosthesis: a limb-saving procedure in children suffering from a malignant bone tumor. *Artificial Organs* **21,** 413-7.
3. **Costerton, J.W., Stewart, P.S. and Greenberg, E.P. (1999).** Bacterial biofilms: a common cause of persistent infections. *Science* **284,** 1318-22.
4. **Gottenbos, B., Grijpma, D.W., Van der Mei, H.C., Feijen, J. and Busscher, H.J. (1999).** Initial adhesion and surface growth of *Pseudomonas aeruginosa* on negatively and positively charged poly(methacrylates). *Journal of Materials Science: Materials in Medicine* **10,** 853-5.
5. **Harkes, G., Dankert, J. and Feijen, J. (1992).** Growth of uropathogenic *Escherichia coli* strains at solid surfaces. *Journal of Biomaterial Science: Polymer Edition* **3,** 403-18.
6. **Harkes, G., Feijen, J. and Dankert, J. (1991).** Adhesion of *Escherichia coli* on to a series of poly(methacrylates) differing in charge and hydrophobicity. *Biomaterials* **12,** 853-60.
7. **Van Wagenen, R.A. and Andrade, J.D. (1980).** Flat plate streaming potential investigations: hydrodynamics and electrokinetic equivalency. *Journal of Colloid and Interface Science* **76,** 305-14.
8. **Gottenbos, B., Van der Mei, H.C. and Busscher, H.J. (1999).** Models for studying initial adhesion and surface growth in biofilm formation on surfaces. *Methods in Enzymology* **310,** 523-34.
9. **Christensen, G.D., Baddour, L.M., Hasty, D.L., Lowrance, J.H. and Simpson, W.A. (1989).** Microbial and foreign body factors in the pathogenesis of medical device infections. In *Infections associated with indwelling medical devices.* Edited by Bisno, A.L. and Waldvogel, F.A. (American Society of Microbiology, Washington DC) p. 27-59.
10. **Nickel, J.C., Costerton, J.W., McLean, R.J. and Olson, M. (1994).** Bacterial biofilms: influence on the pathogenesis, diagnosis and treatment of urinary tract infections. *Journal of Antimicrobial Chemotherapy, supplement A* **33,** 31-41.
11. **Habash, M. and Reid, G. (1999).** Microbial biofilms: their development and significance for medical device-related infections. *Journal of Clinical Pharmacology* **39,** 887-98.
12. **Denstedt, J.D., Wollin, T.A. and Reid, G. (1998).** Biomaterials used in urology: current issues of biocompatibility, infection, and encrustation. *Journal of Endourology* **12,** 493-500.

13. **Gould, I.M. (1999).** A review of the role of antibiotic policies in the control of antibiotic resistance. *Journal of Antimicrobial Chemotherapy* **43,** 459-65.
14. **Walsh, C. (2000).** Molecular mechanisms that confer antibacterial drug resistance. *Nature* **406,** 775-81.